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PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 C.F.R. 1.53 (c).

Docket Number	235.0055 0160	Type a plus sign (+) inside this box >	+

INVENTOR(s)/APPLICANT(s)	
Name (last, first, middle initial)	RESIDENCE (CITY, AND EITHER STATE OR FOREIGN COUNTRY)
Altman, Elliot	Athens, GA
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17302 U.S.PTO
60/519100

TITLE OF THE INVENTION (280 characters max)

BIOTIN-FACILITATED TRANSPORT OF PEPTIDES IN GRAM-NEGATIVE BACTERIA

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ENCLOSED APPLICATION PARTS (check all that apply)

X	Specification	Number of Pages	32		Small Entity Statement
	Drawing(s)	Number of Sheets			Other (specify)

METHOD OF PAYMENT (check one)

X	A check or money order is enclosed to cover the Provisional filing fees	PROVISIONAL FILING FEE AMOUNT	($) 160.00
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government:

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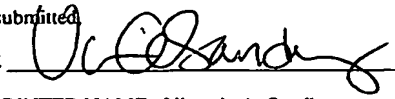
Respectfully submitted,

SIGNATURE  Date Nov 12, 2003

TYPED OR PRINTED NAME Victoria A. Sandberg REGISTRATION NO. 41,287

____ Additional inventors are being named on separately numbered sheets attached hereto.

PROVISIONAL APPLICATION FILING ONLY

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): Elliot Altman and Jennifer R. Walker

Docket No.: 235.0055 0160

Title: BIOTIN-FACILITATED TRANSPORT OF PEPTIDES IN GRAM-NEGATIVE BACTERIA

Assistant Commissioner for Patents
MAIL STOP PROVISIONAL APPLICATION
P.O. Box 1450
Alexandria, VA 22313-1450

We are transmitting the following documents along with this Transmittal Sheet (which is submitted in triplicate):

X PROVISIONAL PATENT APPLICATION including:
- X Specification (32 consecutively numbered pgs, including 20 claims);
- X Provisional Application Cover Sheet (1 pg).

X A check in the amount of $ 160.00 to pay the provisional application filing fee.
___ Verified statement(s) establishing small entity status of this application under 37 C.F.R. 1.9 and 1.27 is/are enclosed.
X An itemized return postcard.
X An Assignment of the invention to The University of Georgia Research Foundation and Recordation Form Cover Sheet (3 pgs).
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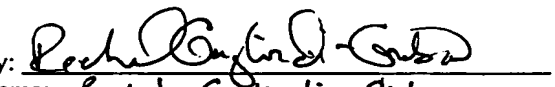
By: 
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(PROVISIONAL TRANSMITTAL UNDER RULE 1.10)

BIOTIN-FACILITATED TRANSPORT OF PEPTIDES IN GRAM-NEGATIVE BACTERIA

Background

The American Medical Association and the Centers for Disease Control and Prevention have become increasingly worried about the dramatic increase in drug-resistance pathogens and as the data shows below the incidence of Gram-negative dug-resistant pathogens is the most problematic, totaling 59.9% of all drug-resistant pathogens that are monitored.

Incidence of antimicrobial-resistant pathogens that are monitored by the CDC.

Antimicrobial-resistant pathogen	Number of cases	Percent of total
Methicillin-resistant *Staphylococcus aureus* (MRSA)	49,247	14.3%
Methicillin-resistant coagulase-negative *Staphylococci* (MRCNS)	29,453	8.5%
Vancomycin-resistant *Enterococcus* spp (VRE)	36,114	10.5%
Ceftazidime, ciprofloxacin/ofloxacin, imipenem, piperacillin, or levofloxacin-resistant *Pseudomonas aeruginosa*	109,165	31.6%
Ceftazidime, cefotaxime, ceftriaxone, imipenem, or meropenem-resistant *Enterobacter* spp	17,252	5.0%
Ceftazidime, cefotaxime, or ceftriaxone-resistant *Klebsiella pneumoniae*	16,834	4.9%
Ceftazidime, cefotaxime, ceftriaxone, ciprofloxacin, ofloxacin, or levofloxacin-resistant *Escherichia coli*	80,729	23.4%
Cefotaxime/ceftriaxone, or penicillin-resistant *Pnuemococci*	6,328	1.8%
TOTAL	345,122	100.0%

Data compiled from the CDC National Nosocomial Infections Surveillance (NNIS) August 2002 Report of Antimicrobial-Resistant Pathogens in Hospitals.

Compounding this problem is the inability of the pharmaceutical industry to trivially generate new antibiotics. Pharmaceutical companies have relied on making derivatives of naturally available compounds for several decades now as evidenced by the multiple generations of new antibiotics from drug classes such as penicillins , cephalosporins, and aminoglycosides. New methods to generate novel antibiotics would be very well received. There has been increasing interest in the development of peptide antibiotics, however, research has focused on the development of novel peptide antibiotics for Gram-positive pathogens due to the problem of peptide uptake by Gram-negative pathogens. This invention allows for the uptake of peptides by Gram-negative bacteria and makes the design of potential peptide antibiotics for us in Gram-negative pathogens possible.

ABSTRACT

In this study, we have shown that biotinylated peptides up to 31 amino acids in length can be taken up by *Escherichia coli* and that uptake is dependent on the biotin transporter. Uptake could be competitively inhibited by free biotin or avidin, and uptake was abolished in *E.coli* that contained a mutation in the biotin transporter. Biotinylated peptides could be used to supplement the growth of a biotin auxotroph and the transported peptides were shown to be localized to the cytoplasm in cell fractionation experiments. The uptake of biotinylated peptides was also demonstrated for two other Gram-negative bacteria, *Salmonella typhimurium* and *Pseudomonas aeruginosa*. This finding may make it possible to create new peptide antibiotics that can be used against Gram-negative pathogens. Researchers have tried to use various moieties to cause the illicit transport of compounds in bacteria and this study demonstrates the illicit transport of the largest known compound to date.

INTRODUCTION

The outer membrane of Gram-negative bacteria functions as a molecular sieve and allows only very small molecules to passively diffuse into the cell. Porins in the outer membrane allow the transport of larger molecules and may be specific or non-specific in their molecular recognition. Non-specific porins such as Omp F, Omp C and Pho E allow the rapid passage of small hydrophilic molecules (Nikaido, 1996; Nikaido and Vaara, 1985). Other porins allow the transport of specific molecules. The peptide permeases, for example, have a specificity for small oligopeptides. The uptake of

oligopeptides is dependent upon size, hydrophobicity and charge (Payne and Gilvarg, 1968; Barak and Gilvarg, 1975; Naider and Becker, 1975).

It is well documented that *Escherichia coli* can not take up large peptides and that the size exclusion limit for porin mediated peptide transport is 650 daltons or the size of a penta- or hexapeptide. The size exclusion limit for peptide uptake in other Gram-negative organisms such as *Salmonella typhimurium* has also been determined and found to be similar to that of *E. coli* (Payne, 1976, 1980; Payne and Smith, 1994). In contrast to Gram-negative bacteria, Gram-positive bacteria can transport much larger peptides. For example, *Lactococcus lactis* has been shown to take up peptides over 18 residues in length or 2,140 daltons in size (Detmers *et al.*, 1998) while *Bacillus megaterium* can transport molecules up to 10,000 daltons in size (Scherrer and Gerhardt, 1971).

This study provides evidence that large biotinylated peptides can be readily transported into Gram-negative bacteria such as *E. coli*. While conducting an *in vivo* screen for randomly encoded peptides which could inhibit the growth of *Staphylococcus aureus*, we performed a test to confirm that potential peptides resulting from the screen would be readily taken up, as expected, by this Gram-positive organism. A biotinylated 10 amino acid peptide was added extracellularly to growing cultures of *S. aureus* and an *E. coli* control, which should not have been able to take up the 1,534 dalton peptide. The synthetic peptides had been biotinylated so they could be easily visualized on Western blots using a neutravidin horseradish peroxidase conjugate. Surprisingly, we found that the peptide was taken up by both *S. aureus* and *E. coli* within 5 minutes of incubation. This observation appeared to contradict the known size exclusion limit of *E. coli* and suggested that the biotinylation of peptides may allow for peptide uptake to occur via the biotin transport system.

MATERIALS AND METHODS

Bacterial strains

E. coli MG1655 (wild-type F- λ-), *E.coli* S1036 (Δ*bio*61 *bioP*98 (up promoter) *recA*1 *thi rpsL* λ *b*515 *b*519 *galq*6 *red*270 *cI*857), *E. coli* S1039 (*birB*ts13 Δ*bio*61 *bioP*98 (up promoter) *recA*1 *thi rpsL* λ *b*515 *b*519 *galq*6 *red*270 *cI*857), *E. coli* SA291 (*rpsL his* Δ(*gal-chlA*)), *Pseudomonas aeruginosa* ATCC9721, *S. typhimurium* LT2, and *S. aureus* ATCC25923 were the bacterial strains used in this study. *E. coli* S1036 and S1039 were derived from SK121 which is a derivative of SK98 (Ketner and Campbell, 1975) and contains a mutation in the λ prophage that enables SK121 to grow at 43°C (Allan Campbell, personal communication).

Media

Rich LB and minimal M9 media as described by Miller, 1972 was used for *E. coli* MG1655 and *S. typhimurium* cultures. Rich LB and minimal media as described by Gilleland *et al.*, 1974 was used for *P. aeruginosa*. Tryptic soy broth and minimal media as described by Mah *et al.*, 1967 was used for *S. aureus*. Rich LB and minimal media as described by Campbell, 1961 was used for *E. coli* S1036, S1039 and SA291.

Peptides and reagents

The randomized biotinylated peptides XXXX[KBtn]XXXXA (10 amino acids) and XXXXXXXXXXXXXXX[KBtn]XXXXXXXXXXXXXXA (31 amino acids) were synthesized by Sigma Genosys, where A denotes the L-amino acid alanine, X denotes an equimolar mixture of all 20 natural L-amino acids, and KBtn denotes the L-amino acid lysine to which biotin has been attached. The average molecular weight of the 10 and 31 amino

acid peptides were determined to be 1,534 and 3,904 daltons, respectively, using an Applied Biosystems Voyager System 1105 mass spectrometer. This was in very close agreement with the theoretical molecular weights for the 10 and 31 amino acid peptides which were 1,517 and 3,947 daltons, respectively. Biotin, thiamine, avidin, and bovine serum albumin were purchased from Sigma. NeutrAvidin Horseradish Peroxidase Conjugate and SuperSignal West Dura Extended Duration Chemiluminescent Substrate were purchased from Pierce.

Uptake assays

Cell cultures were started from rich overnights, washed once using minimal media, diluted into minimal media, and then incubated at 37°C overnight until the cultures had reached saturation. Cultures were diluted again in minimal media and incubated until they reached an OD_{550} of 0.5. The 10 and 31 amino acid randomized biotinylated peptides were added to the media at a concentration of 1 μg per mL of culture. After addition of the peptide to the culture, 1 mL aliquots were extracted at time intervals up to an hour, washed of extracellular peptide using fresh minimal media, then boiled with SDS-PAGE gradient sample buffer. Samples were run on a 10-16% tricine gradient gel (Schagger and von Jagow, 1987) and transferred to nitrocellulose membranes. The resulting Western blots were treated with NeutrAvidin Horseradish Peroxidase Conjugate and SuperSignal West Dura Extended Duration Chemiluminescent Substrate. The membranes were incubated for 5-10 minutes then exposed to autoradiography film for 1 minute. Bands on the film were quantified using the AlphaEase 5.5 Densitometry Program from Alpha Innotech.

To test for competitive inhibition, the 31 amino acid biotinylated peptide and an equimolar and ten times equimolar amount of biotin, thaimine, avidin, or BSA was

added to midlog cell cultures. After 10 minutes of incubation, 1 ml samples were removed and analyzed as described above for the general uptake assay.

Cell fractionation

The 31 amino acid biotinylated peptide was added at a concentration of 1 μg per mL to *E. coli* MG1655 cells that had been grown to an OD_{550} of 0.5 in minimal maltose media to allow for the induction of the maltose binding protein which served as one of the fractionation controls. Cultures were then subjected to periplasmic shock as described by Ames *et al.*, 1984 to isolate the periplasmic fraction. The remaining cell pellet was then further fractionated using the method described by Altman *et al.*, 1983 to prepare cytoplasmic and membrane fractions with one modification. Cytoplasmic proteins were precipitated by adding trichloroacetic acid at a final concentration of 5% w/v to the cytoplasmic fraction. The precipitate was then centrifuged at 4°C, 50,000 rpm for 30 minutes to pellet the cytoplasmic proteins. The periplasm, cytoplasm, and membrane samples were analyzed using a 10-16% tricine gradient gel and Western blotted as described above for the uptake assays.

RESULTS

Biotinylated peptides up to 31 amino acids in length can be taken up by *E. coli*

We initially tested the ability of *E. coli* and *S. aureus* to import a 10 amino acid biotinylated peptide. Randomized peptides were used as opposed to peptides with a specific sequence in order to avoid nonspecific uptake that might be caused by certain amino acid sequences. Peptide was added to mid-log cultures of bacteria which were

allowed to incubate for time intervals up to 60 minutes in duration. Samples were removed at specific times, pelleted, washed to remove any peptide in the media that had not been taken up by the cells, and then analyzed as described in Materials and Methods. As shown in Figure 1, both *E. coli* and *S. aureus* readily imported the 10 amino acid biotinylated peptide. Using densitometry, we determined that up to 75% of the peptide was imported within the first 5 minutes of incubation. To determine whether the import, which was arguably due to biotinylation in *E. coli*, was limited to smaller peptides, we also tested whether a much larger 31 amino acid biotinylated peptide could be imported in *E. coli* and *S. aureus*. As with the 10 amino acid biotinylated peptide, the 31 amino acid biotinylated peptide was also taken up by both *E. coli* and *S. aureus* (data not shown).

The uptake of biotinylated peptides in *E. coli* can be competitively inhibited by biotin or avidin

Given that peptides larger than six amino acids can not be taken up by *E. coli*, the obvious interpretation of our results was that biotin was the mechanism by which this unexpected uptake was occurring. To test this assumption, we conducted a competition experiment in both *E. coli* and *S. aureus* using biotin. We rationalized that since large peptides can be readily taken up by Gram-positive bacteria such as *S. aureus*, biotin should have no competitive effect. However, in *E coli*, if the uptake was due to biotin, then free biotin should be able to competitively block uptake. Figure 2 shows that this is indeed the case. The uptake of biotinylated peptides could be blocked in *E.coli* by the addition of biotin whereas biotin had no effect on the uptake of biotinylated peptides in *S. aureus*. Additionally, we showed that the competitive inhibition in *E. coli* was specific to biotin and the use of another similarly sized vitamin, thiamine, had no effect.

Because avidin is known to tightly bind biotin (Green, 1963), we also tested whether avidin would be able to competitively inhibit the uptake of biotinylated peptides in *E. coli*. Figure 3 shows that avidin could competitively inhibit the uptake of biotinylated peptides in *E. coli*, but that the use of another similarly sized protein, bovine serum albumin, which is routinely used in *in vitro* studies, had no effect.

The uptake of biotinylated peptides in *E. coli* is dependent on the biotin transport system

The biotin transport system in *E. coli* has been well characterized and mutants that inactivate the biotin transporter, *birB/bioP*, are available (Eisenberg *et al.*, 1975; Campbell *et al.*, 1980). If the import of biotinylated peptides in *E. coli* were indeed due to the biotin transport system, then *birB* mutants should not be able to take up biotinylated peptides. Figure 4 shows that this is the case. A wild-type $birB^+$ strain was able to take up biotinylated peptide, while an isogenic $birB^-$ mutant strain was not.

Biotinylated peptides can be used to fulfill the growth requirements of an *E. coli* biotin auxotroph.

To further demonstrate that biotinylated peptides were truly taken up by *E. coli*, we tested whether a biotinylated peptide could be used instead of biotin to fulfill the growth requirement of an *E. coli* biotin auxotroph in minimal media. Figure 5 shows that an *E.coli* biotin auxotroph grows as well in media supplemented with biotinylated peptide as it does in media supplemented with biotin.

Cell fractionation studies show that biotinylated peptide can be detected in the cytoplasm of *E. coli*.

To demonstrate biochemically that biotinylated peptides were taken up by *E.coli*, we performed cell fractionation studies where periplasmic, cytosolic, and membrane fractions were prepared from cultures to which biotinylated peptide had been added. Figure 6 shows that the biotinylated peptide localized to both the cytoplasmic and membrane fractions. Of the peptide that could be detected, 66% was found in the membrane fraction and 33% was found in the cytoplasmic fraction. To verify that the cell fractionation studies had been done correctly, we used the same cell fractions to visualize the GroEL and MBP proteins which are known to localize to the cytoplasm and periplasm, respectively. GroEL was found primarily in the cytoplasmic fraction, while MBP was found primarily in the periplasmic fraction. GroEL's distribution was 93% in the cytoplasm and 7% in the membrane, while MBP's distribution was 95% in the periplasm, 3% in the membrane and 2% in the cytoplasm (data not shown).

Biotinylated peptides can be taken up by other Gram-negative bacteria

Given our findings in *E. coli*, we also wanted to test whether biotinylated peptides could be transported by other Gram-negative bacteria. We found that both the 10 and 31 amino acid biotinylated peptides could be readily transported by both *S. typhimurium* and *P. aeruginosa*. Figure 7 shows the uptake of the 31 amino acid biotinylated peptide by *S. typhimurium* and *P. aeruginosa*.

DISCUSSION

It has been well established that Gram-negative bacteria such as *E. coli* can only take up very small peptides that are six amino acids or less in size. In this study, we have shown that biotinylation can facilitate the uptake of peptides up to 31 amino acids in length by *E. coli* and that transport is dependent on the biotin transporter, *birB/bioP*, and can be competitively inhibited by free biotin or avidin. We also demonstrated that biotinylated peptide could be used to supplement the growth of a biotin auxotroph and that the biotinylated peptide was localized to the cytoplasm in cell fractionation studies. What is known about biotin function in *E. coli* is consistent with our finding that biotin can be used to facilitate the uptake of peptides via the biotin transporter in *E. coli*.

Biotin can be synthesized as well as transported by *E. coli* and the genes involved in biotin biosynthesis and transport are repressible by biotin (Guha *et al.*, 1971). Biotin's transport system is regulated independent of the biosynthetic pathway (Pai, 1973). *E. coli* readily imports the vitamin when it is available and concomitantly represses biotin synthesis. Biotin uptake is specific, energy dependent, and can accumulate against a concentration gradient (Prakash and Eisenberg, 1974; Piffeteau *et al.*, 1982; Piffeteau and Gaudry, 1985). Maximum uptake is observed during exponential growth phase (Piffeteau *et al.*, 1982) and glucose has been shown to increase biotin uptake slightly. The rate of biotin uptake has also been shown to increase proportionally to the amount of extracellular biotin that is available (Prakash and Eisenberg, 1974).

The first biotin transporter mutant was discovered by Campbell *et al.*, 1972. They termed the mutant *bir* for biotin retention and showed that the mutant abolished the ability of *E. coli* to take up biotin. Eisenberg *et al.*, 1975 isolated an independent mutant that abolished biotin uptake which they termed *bioP*. Campbell *et al.*, 1980

renamed their original *bir* mutant *birB* and showed that *birB* and *bioP* mutants were identical via genetic mapping experiments.

It is surprising that the biotin transport system can be used to facilitate the uptake of large peptides. Biotin has a molecular weight of 244, making it relatively small in comparison to a 10 amino acid biotinylated peptide with an average molecular weight of 1,534 or a 31 amino acid biotinylated peptide with an average molecular weight of 3,904. Clearly the biotin uptake system must be flexible since it can accommodate larger molecules. Our finding that 33% of the biotinylated peptide localized to the cytoplasm and 66% of the peptide localized to the membrane is consistent with such a model. Some of the biotinylated peptide was able to completely pass through the biotin transporter while a significant fraction remained in the membrane.

There is contradictory evidence with regards to how biotin's structure affects its ability to be taken up by *E. coli*. Prakash and Eisenberg, 1974 stated that while the ureido ring of biotin must be intact for uptake, modification of the side chain has little effect. However, Piffeteau *et al.*, 1982 suggested that modifications to the side chain of biotin could drastically affect biotin's ability to be taken up and that the carboxyl group on the side chain is essential for biotin uptake. In the biotinylated peptides used in this study, the biotin carboxyl group is joined to the amino group of lysine via an amide bond and thus the carboxyl group of biotin is not available for recognition. This fact supports Prakash and Eisenberg's argument that the side chain of biotin does not affect uptake. Our data further suggests that it is indeed the ureido ring that is required for recognition and uptake.

The fact that biotinylation can facilitate the uptake of very large peptides by Gram-negative bacteria represents the illicit transport of the largest known compound

to date. Illicit transport has been defined as the entry of compounds into cells through the use of transport systems designed for other substrates (Ames *et al.*, 1973). There are numerous examples of the use of peptide permeases to facilitate the uptake of small antibacterial peptides or antibiotics that have been coupled to di- or tripeptides (Ames *et al.*, 1973; Fickel and Gilvarg, 1973; Atherton *et al.*, 1980; Staskawicz and Panopoulos, 1980; Morley *et al.*, 1983). Additionally, researchers have used various siderophores that are involved in iron uptake to facilitate the transport of antibiotics (Luckey *et al.*, 1972, Miller *et al.*, 1991; Wittmann *et al.*, 2002). All of these compounds are much smaller than the 10 and 31 amino acid peptides that we have found to be transported in this study via biotinylation.

Interestingly, biotinylated molecules are currently being investigated for drug delivery in mammalian cells. Avidin drugs that bind to biotinylated vectors are being used to promote delivery across the blood brain barrier (Bonfils *et al.*, 1992; Pardridge, 2002; Song *et al.*, 2002) while antitumor toxins or imaging agents coupled to streptavidin are being delivered using biotinylated antibodies (Press *et al.*, 2001; Hussey and Peterson, 2002). Biotinylation has also been shown to promote the delivery of polyethylene glycol camptothecin conjugates into human ovarian carcinoma cells (Minko *et al.*, 2002) and increase the cellular uptake of polyethylene glycol TAT nonapeptide conjugates into human Caco and CHO cells (Ramanathan *et al.*, 2001).

Our finding that biotinylated peptides can be taken up by Gram-negative bacteria such as *E. coli, S. typhimurium* and *P. aeruginosa,* represents an intriguing possibility for the development of antibacterial peptides. Given the abundance of naturally occurring antibacterial peptides and the increased interest in designing new synthetic peptide drugs, researchers have been trying to develop novel peptide antibiotics that can inhibit the function of key intracellular targets identified through

genomics. Researchers have been focusing on Gram-positive bacteria where the uptake of large peptides is not problematic. The use of biotinylated peptides may make it possible to use this same approach to develop antibacterial peptides that can target Gram-negative bacteria.

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FIGURE LEGENDS

Figure 1. Uptake of a 10 amino acid biotinylated peptide by *S. aureus* (A) and *E. coli* (B). The biotinylated peptide was added to mid-log cultures, aliquots were taken at different time interval and analyzed as described in Materials and Methods. Peptide only and cell only samples were included as controls.

Figure 2. Effect of biotin on the uptake of a 31 amino acid biotinylated peptide in *E. coli* and *S. aureus*. Equimolar and 10X equimolar amounts of biotin or thiamine and the biotinylatyed peptide were added to mid-log cultures. Aliquots were taken after 10 minutes of incubation and analyzed as described in Materials and Methods.

Figure 3. Effect of avidin on the uptake of a 31 amino acid biotinylated peptide in *E. coli*. Equimolar and 10X equimolar amounts of avidin or bovine serum albumin and the biotinylatyed peptide were added to mid-log cultures. Aliquots were taken after 10 minutes of incubation and analyzed as described in Materials and Methods.

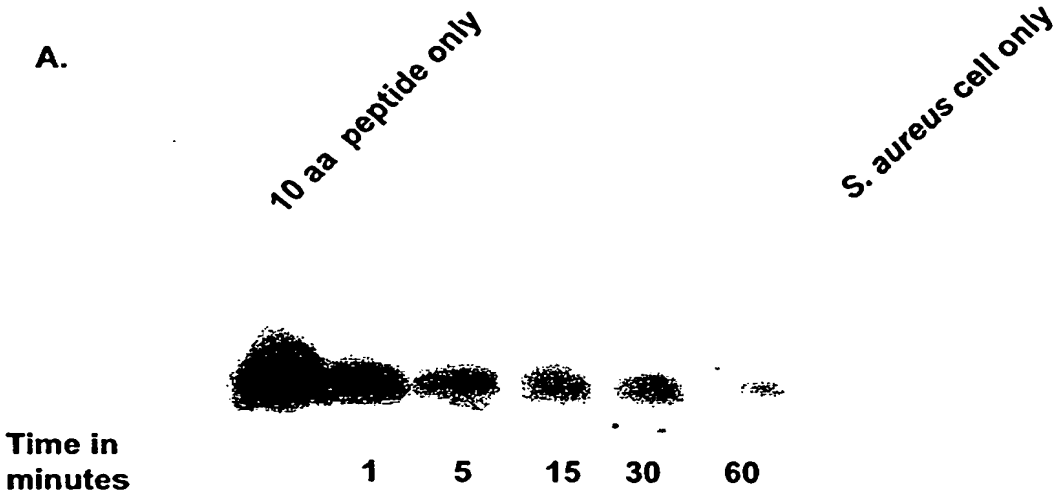
Figure 4. Effect of a *birB*⁻ mutation on the uptake of a 31 amino acid biotinylated peptide in *E. coli*. The biotinylated peptide was added to mid-log cultures of *birB*⁺ and *birB*⁻ cells. Aliquots were taken after 10 minutes of incubation and analyzed as described in Materials and Methods. Peptide only and cell only samples were included as controls.

Figure 5. Growth of an *E. coli bio* auxotroph on minimal media supplemented with biotin or equimolar amounts of biotinylated peptides. The *E. coli* SA291 *bio* auxotrophic strain was grown in minimal media at 37°C with either no supplement (Δ), 1 μg/mL biotin (□), or equimolar amounts of the 10 (○) and 31 (◊) amino acid biotinylated peptides. Aliquots were removed at 12 hour intervals and the OD_{550} was determined.

Figure 6. Localization of biotinylated peptide in *E.coli*. Biotinylated peptide was added to mid-log cultures and the cells were fractionated into periplasmic, cytoplasmic, and membrane samples and analyzed as described in Materials and Methods. A whole cell sample was included as a control.

Figure 7. Uptake of a 31 amino acid biotinylated peptide by *S. typhimurium* (A) and *P. aeruginosa* (B). Biotinylated peptide was added to mid-log cultures, aliquots were taken at different time intervals and analyzed as described in Materials and Methods. Peptide only and cell only samples were included as controls.

Figure 1


A.
10 aa peptide only
S. aureus cell only
Time in minutes
1
5
15
30
60

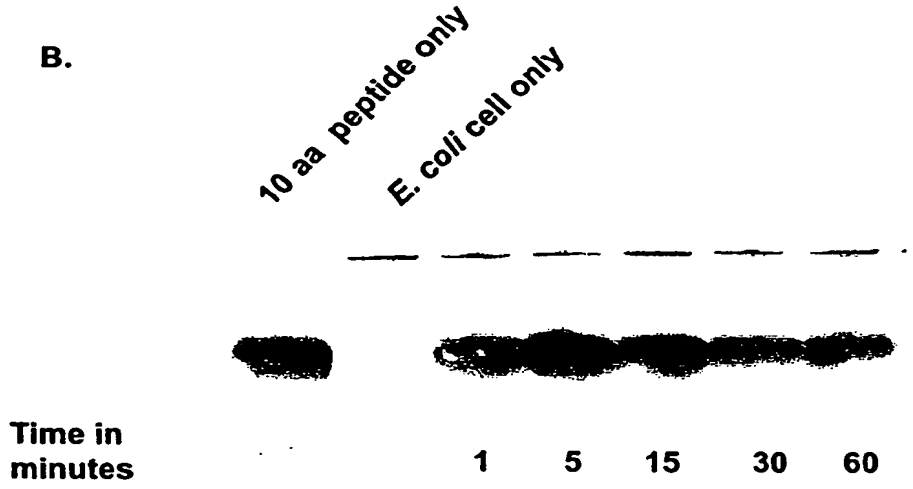

B.
10 aa peptide only
E. coli cell only
Time in minutes
1
5
15
30
60

Figure 2

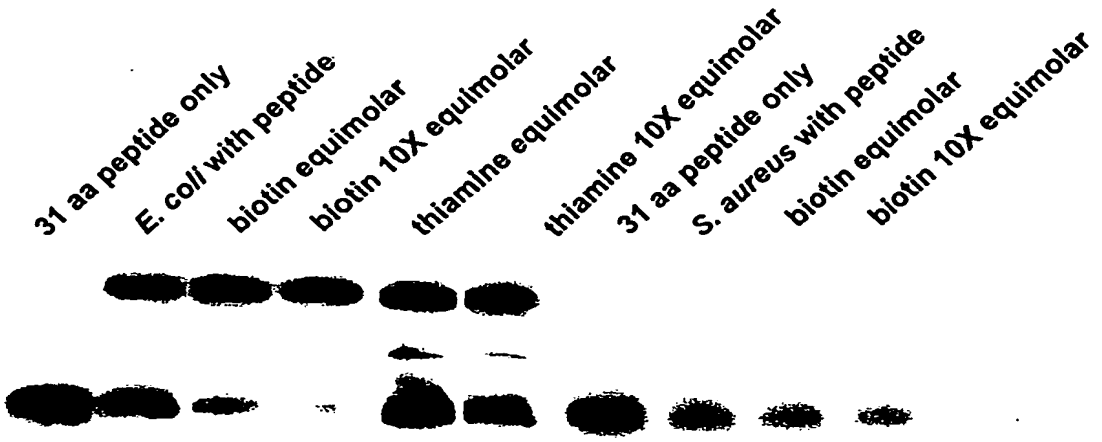

31 aa peptide only
E. coli with peptide
biotin equimolar
biotin 10X equimolar
thiamine equimolar
thiamine 10X equimolar
31 aa peptide only
S. aureus with peptide
biotin equimolar
biotin 10X equimolar

Figure 3

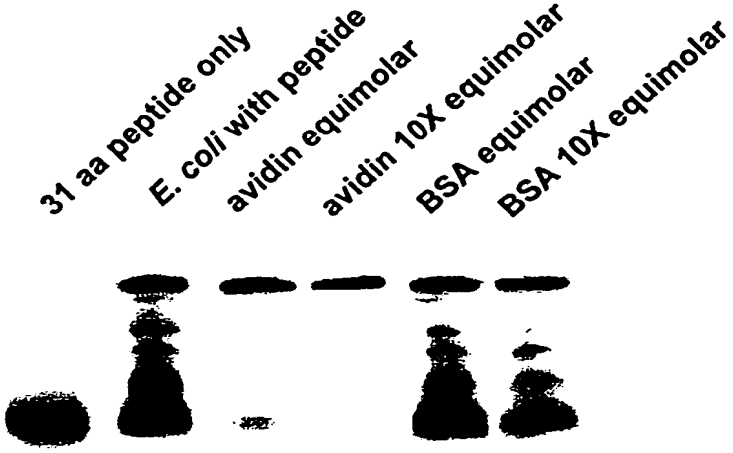

31 aa peptide only
E. coli with peptide
avidin equimolar
avidin 10X equimolar
BSA equimolar
BSA 10X equimolar

Figure 4

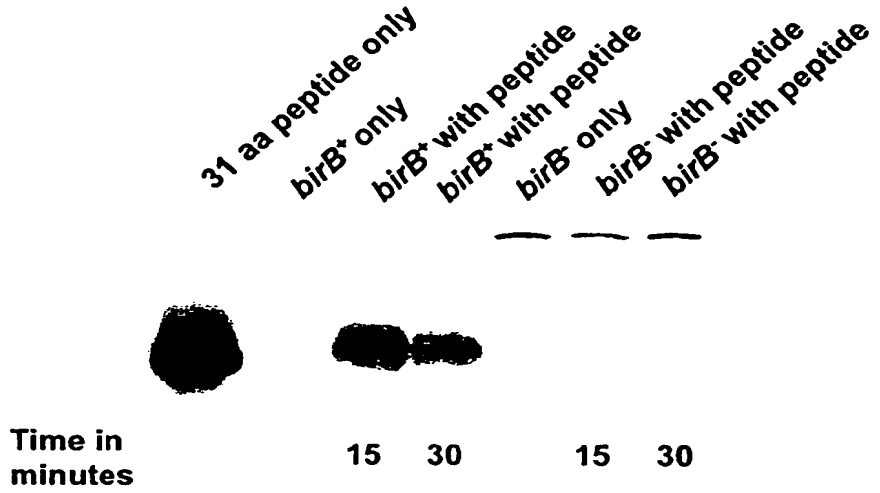

31 aa peptide only
birB+ only
birB+ with peptide
birB+ with peptide
birB- only
birB- with peptide
birB- with peptide
Time in minutes
15
30
15
30

Figure 5

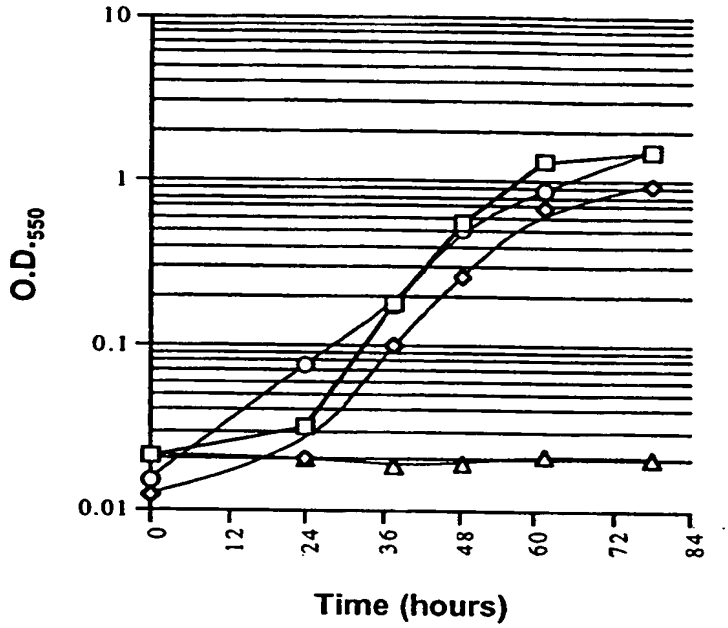


Figure 6

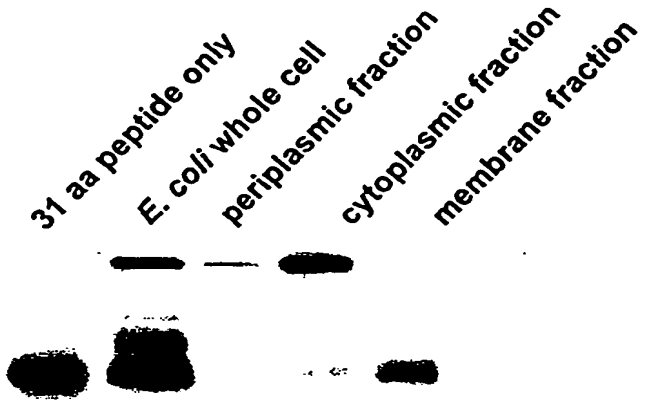
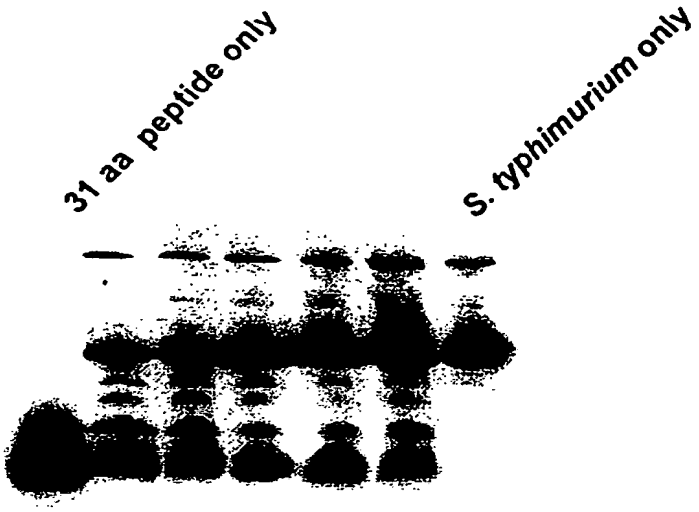

31 aa peptide only
E. coli whole cell
periplasmic fraction
cytoplasmic fraction
membrane fraction

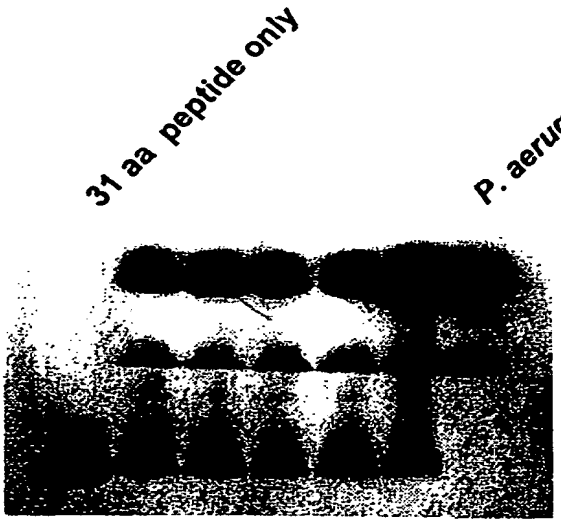
Figure 7

A.



Time in minutes 1 5 15 30 60

B.



Time in minutes 1 5 15 30 60

Compounds whose transport into Gram-negative bacteria can be facilitated by derivitization with a biotin moiety include, for example, naturally occurring or synthetic peptides, peptidomimetics, and derivatives and conjugates thereof.

A peptidomimetic is a compound containing non-peptidic structural elements that is capable of mimicking or antagonizing the biological action(s) of a natural parent peptide. A peptidomimetic typically does not possess classical peptide characteristics such as enzymatically scissile peptidic bonds. See "Glossary of Terms used in Medicinal Chemistry, a publication of the International Union of Pure and Applied Chemistry (IUPAC) (IUPAC Recommendations 1998).

Biotinylated peptides or peptidomimetics may themselves be bioactive and/or they can be conjugated to a bioactive compound such as a therapeutic, diagnostic or imaging agent to facilitate delivery of the bioactive compound to Gram-negative bacteria. The compound that is conjugated to the peptide can any type of compound. The transport mechanism of the invention can be advantageously employed to reliably target and deliver known and newly discovered drugs to Gram-negative bacteria via biotinylation of the drug. In some instances, biotin-mediated transport can serve as a secondary membrane transport system for a bioactive compound that already makes use of a different transmembrane transport system, thereby increasing efficacy by improving delivery to the target cell. In other instances, the bioactive compound can contain a targeting moiety, such as a particular receptor ligand, that is specific for Gram-negative bacteria, in addition to a biotin moiety for facilitated transport once the compound is in contact with the cell membrane.

The present invention is illustrated by the preceding examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

The complete disclosures of all patents, patent applications including provisional patent applications, and publications, and electronically available material (e.g., GenBank amino acid and nucleotide sequence submissions) cited herein are incorporated by reference. The foregoing detailed description and examples have been provided for clarity of understanding only. No unnecessary limitations are to be understood therefrom.

What is claimed is:

1. A method for introducing a bioactive compound into a Gram-negative bacterial cell, the method comprising contacting the cell with a biotinylated compound, wherein the bioactive compound comprises a bioactive peptide, peptidomimetic, derivative or conjugate thereof.

2. The method of claim 1 further comprising covalently linking a biotin moiety to the compound to yield the biotinylated compound.

3. The method of claim 1 wherein the biotinylated compound comprises a biotin moiety covalently linked to the compound through a biotin carboxyl group.

4. The method of claim 1 wherein the compound comprises a peptide or peptidomimetic comprising least 2 amino acids.

5. The method of claim 1 wherein the compound comprises a peptide or peptidomimetic comprising at least 5 amino acids.

6. The method of claim 1 wherein the compound comprises a peptide or peptidomimetic comprising at least 10 amino acids.

7. The method of claim 1 wherein the compound comprises a peptide or peptidomimetic comprising at least 20 amino acids.

8. The method of claim 1 wherein the compound comprises a peptide or peptidomimetic comprising between 10 and 31 amino acids.

9. The method of claim 1 wherein the compound comprises a peptide or peptidomimetic comprising between 10 and 50 amino acids.

10. The method of claim 1 wherein the Gram-negative bacterial cell is a cell of the genus

Escherichia, *Salmonella*, or *Pseudomonas*.

11. The method of claim 1 wherein the Gram-negative bacterial cell is an *E. coli* cell, a *S. typhimurium* cell, or a *P. aeuriginosa* cell.

12. The method of claim 1 wherein the Gram-negative bacterial cell comprises a biotin transporter molecule, and wherein the biotinylated compound is introduced into the cell through the biotin transporter molecule.

13. The method of claim 13 where the biotin transporter molecule is a *birB/bioP* receptor.

14. The method of claim 1 wherein the compound comprises a therapeutic, diagnostic or imaging agent.

15. The method of claim 15 wherein the compound further comprises a targeting moiety that specifically targets a Gram-negative bacterial cell.

16. The method of claim 1 wherein the compound comprises an antibiotic peptide or peptidomimetic.

17. The method of claim 1 wherein the compound, prior to biotinylation, comprises a naturally occurring peptide.

18. The method of claim 1 wherein the compound, prior to biotinylation, comprises a synthetic peptide.

19. The method of claim 1 wherein the biotinylated compound, when introduced into the cell, inhibits the growth of the cell.

20. The method of claim 1 wherein the biotinylated compound, when introduced into the cell, causes the death of the cell.

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